Cultured Neuron Probe

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General Introduction

Our aim is to develop a cultured neuron probe. This consists of a silicon structure into which individual dissociated neurons can be placed, and which can be inserted into an intact nervous system. Furthermore, within the structure each neuron is in close proximity to an electrode, by means of which it can be stimulated, or its activity can be recorded, through electrical leads which connect to external electronics. It is hoped that neurons in the probe will synaptically integrate with the host nervous system, to provide a highly specific, relatively non-invasive, two-way communication channel. If this occurs, the methodology has important possibilities for neural prostheses. The goal of this project is to perform initial experiments to establish the feasibility of communication by means of a neuron probe. The tissue we have chosen in which to initially implant the probe is the rat hippocampus. If initial studies are successful, probes will be designed and implanted for communication with sensorimotor cortex.

The neuron probe we plan to fabricate will be made of micromachined silicon and will have sixteen electrodes: one conventional electrode to detect activity during placement of the probe, and fifteen within wells into which neurons will be placed. Its configuration will be similar to that of passive multielectrode probes which have been developed. It will be implanted when the cultured neurons are very young, and after a time of weeks it is hoped that they will have survived and made two-way synaptic connections. By stimulation and recording in the host and probe, we will test for the existence of such connections. An essential feature of the experiments is that the viability of the implanted neurons will be independently determined over time by stimulating them and recording their resulting action potentials.

Introduction

During this quarter two new postdoctoral fellows have joined the project, Steven Potter, who is developing hippocampal slice experiments, and Michael Maher, who is working on neurochip studies.

The major progress during the quarter has been:

1. Implementation of a new culture system for hippocampal cells. This system avoids the complexity of coculturing with glia, and typically produces better neuron growth and longer term survival.

2. Improved Di-I staining of dissociated hippocampal neurons, using a sucrose solution and DMF solvent.

3. Successful development of an electrical connection from the pads of a neuron probe to the pins of a skull plug, using 16 25-micron gold wires insulated with MDX4-4210.

4. A new batch of dummy probes was made, to provide a total of more than 40 probes for preliminary studies at Rutgers. Strength tests were performed, which indicated the probes were both strong and flexible.

5. The first neuron probes with electrodes were successfully fabicated.

6. Work continued on studying the growth of test cultures of SCG's on neurochips. Some evidence has been accumulated that flat-bottomed wells, modified from the original design, may be needed to achieve a high probability of cell attachment and growth. Furthermore, there have been instances of cells escaping from wells, indicating a problem for further study.

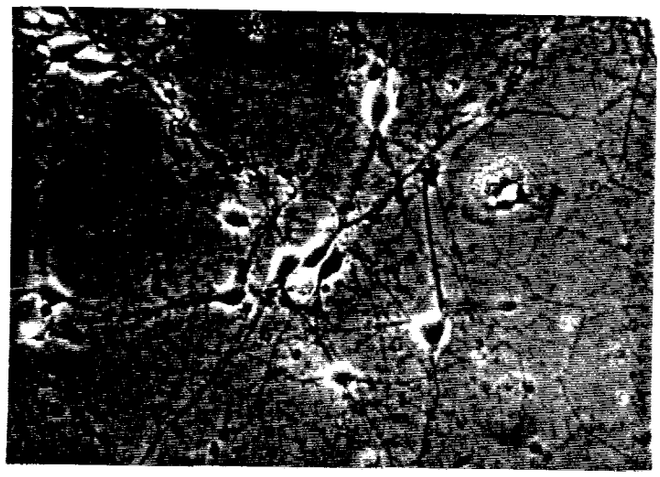
7. Hippocampal cells were loaded into dummy probes successfully at Rutgers, and a staining system for ascertaining whether cells are alive or dead has been used. In the Rutgers culture system, cells remained live for up to 72 hours, but did not extend processes. Possible causes are being investigated.

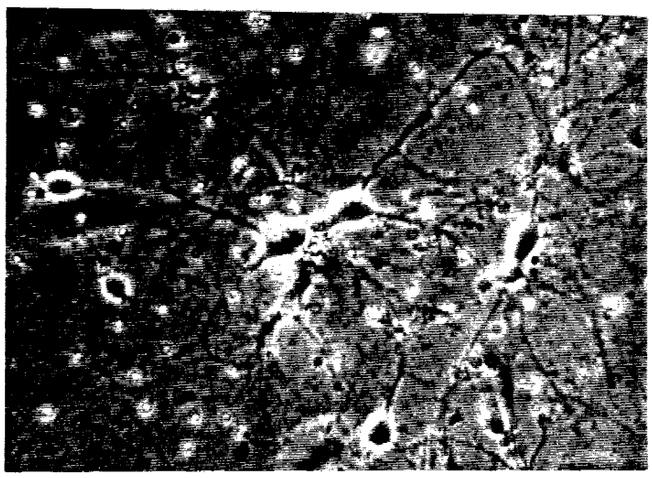
8. Experiments were done with dummy probes placed in rat hippocampus to study both short and long-term damage effects by sectioning fixed tissue and staining with the Gallyas "dark neuron" stain and with activated microglia stain.

In Vitro Studies

1. Cell culture

A major improvement in the hippocampal culture system was made. A new medium, recently announced by Gibco, called neurobasal medium, with a set of additives called B27 supplement, was tried. The supplement was developed by Greg Brewer at Southern Illinois University Medical School, from lengthy trial and error studies. The goal of his work was to eliminate the need for biochemical support from neighboring glia in a serum-free culture system like the Banker system we have been using. Our trials indicate that the Gibco medium and supplement are better than our glia were. Below are two phase micrographs showing healthy pyramidal cells in B27 cultures at 18 days (above) and 25 days (below) days in vitro.





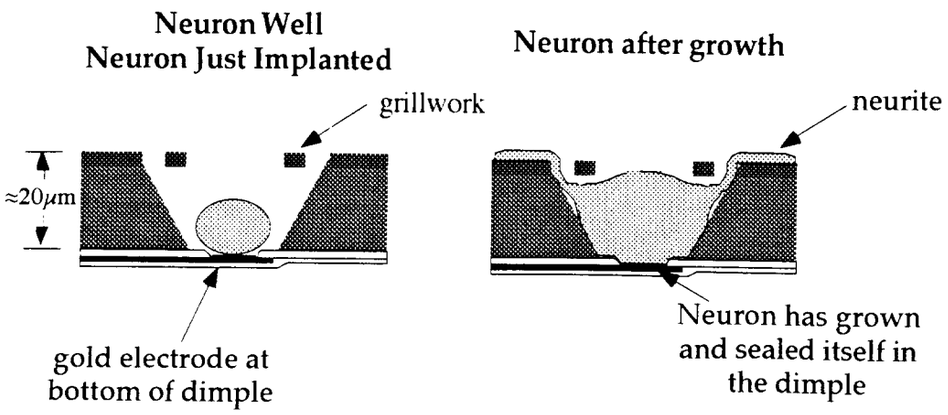
For more intense Di-I staining of dissociated cells, trials have been made using isotonic sucrose as the staining medium. Since Di-I is soluble in this medium, precipitation into microcrystals and poor staining were avoided. In addition, a strong stock solution was used with DMF as the Di-I solvent. The results have been encouraging, and ongoing experiments are planned for optimizing the staining time and the concentration of Di-I stock solution.

2. Neurochip Experiments

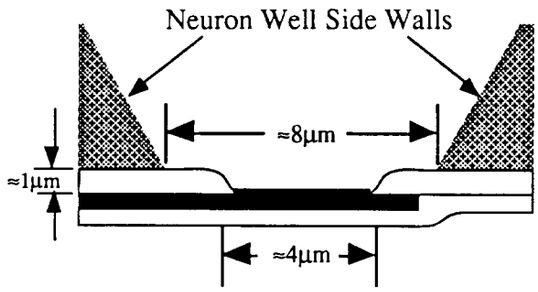
Over the past three months we have become sufficiently adept at putting neurons into wells that all 16 wells of a neurochip can be filled in approximately 20 minutes. However, neuron growth out of the wells was very poor. We hypothesized that the trouble may lie in the bumpy surface of the bottom of the well; it is possible that neurons do not adhere well, and therefore don't attach to the surface. To test this possibility, we have tried three things: etching out the silicon oxide on the bottom of the wells of the older neurochips with HF, attempting further to grow cultures on dummy probes, and starting experiments on a new batch of neurochips with flat bottoms.

Using old neurochips which had been treated with another HF etch to flatten the bottom, we obtained some encouraging results. The first two attempts were not very successful; however, on the third and fourth tries, there was growth out of 8 wells on the neurochip after a day. We also had successes growing cultures on dummy probes, which have flat bottomed wells, with as many as 11 out of 15 wells showing growth after a day in culture. These experiments were meant as an interim project to be pursued until flat-bottomed neurochips became available.

The figures below illustrate the ideal case we originally envisioned, with a "dimple" at the bottom of the well facilitating a seal between the neuron and the electrode.

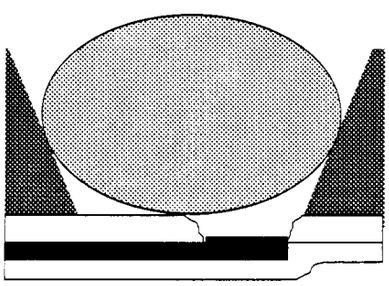


Dimple Magnified



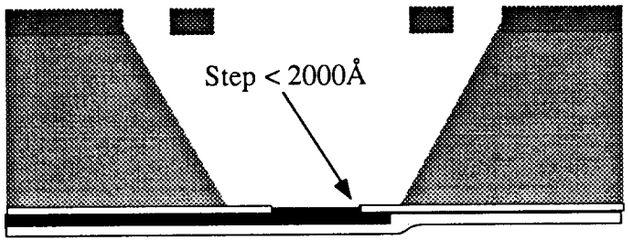
As designed, the dimple should provide an ideal point for the neuron to attach. Unfortunately, life is seldom ideal. In practice, the dimple is typically 1 to 2 μm off center. With the symmetry no longer present, the neuron, centered in the well by the side walls, does not sink snugly into the dimple. Instead, the dimple acts to reduce the surface on to which neuron can attach and creates a "rough" floor, as shown below.

Offset Dimple



With this theory proposed, it was decided to fabricate some chips that would have flat bottoms. This did not require any additional steps be added to the original process, in fact, it removed several deposition stages, resulting in a quicker fab time. The new wells are shown below.

Flat Bottomed Neuro-well



We now have a new supply of neurochips designed in such a way that the bottom surface of each well is completely flat. On each chip, half of the wells have large gold electrodes forming the entire bottom surface of the well; the other half have smaller electrodes. We will thus be able to compare the two configurations to see which gives the best recording and stimulation. Recently we have been trying to establish long-term cultures on these flat-bottomed chips. Results so far, in a small number of trials, have been mixed, with a maximum of 7 out of 16 wells showing growth. However, experiments are continuing

Two further problems in growing neuronal cultures in neurochip wells have emerged: difficulty in determining whether neurite growth seen on the neurochip is in fact coming from neurons in the wells, and the possibility that neurons put into wells do not remain there. Definitive recognition of neurite growth from wells is difficult for two reasons. First, the patterned top surface of the neurochip, an artefact of the etching procedure, makes it very difficult to see fine neurites. Upcoming batches of neurochips will be made with boron-doped epi-layers that are stress-relieved with germanium to eliminate this problem.

Secondly, the plating procedure often results in a large population of neurons in the basin outside of the immediate area of the wells; after a day or two in culture, neurites from these neurons invade the well area and cannot be

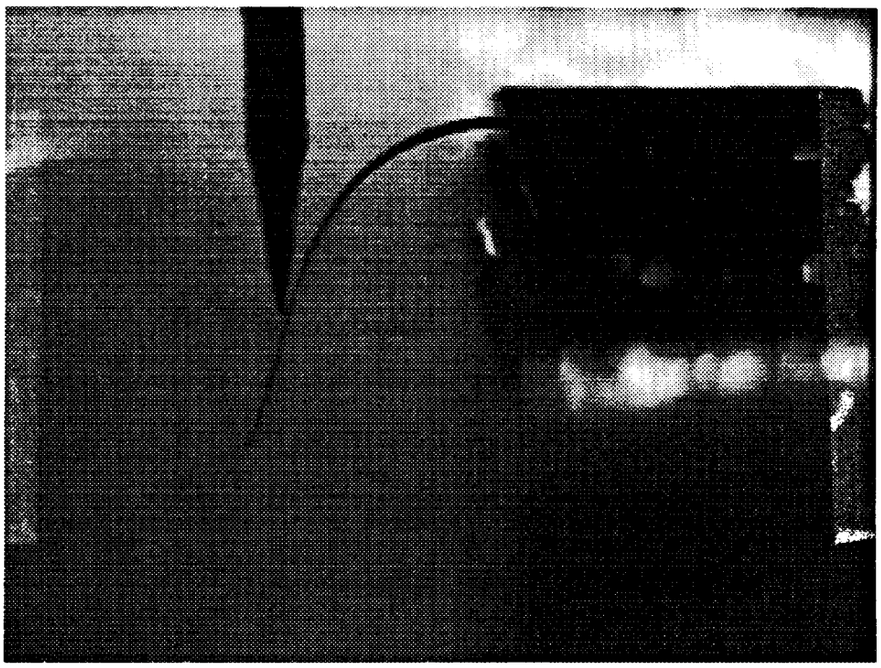
distinguished from neurites coming from the wells. We have tried several tactics to deal with this difficulty, including "vacuuming up" excess neurons from the basin after loading the wells, coating most of the basin surface with Sylgard to limit neuronal adhesion outside of the well area, delimiting an area around the wells with walls of silicone glue to prevent neurite growth from most of the basin, and thorough rinsing of the chip with medium following well loading in an attempt to remove any neurons that have not yet settled. A combination of the silicone glue walls and rinsing with medium should be effective in limiting the number of extraneous neurons in the basin.

Another new problem that has arisen is the phenomenon of neurons escaping from the wells into which they were placed. It is well known that neuron cell bodies in culture can move around, presumably by means of tension exerted by the neurites; however, it was hoped that neurons would be trapped in the wells by the grillwork at the top of the wells. Two lines of evidence suggest that neurons have not all remained in wells. First, in a few cases, neurons have appeared in the well area several days after plating, well after all neurons should have settled down and all neurons near the well area were removed. Second, with the new, flat-bottomed batch of neurochips, it is actually possible to see what is inside the wells (because of the large surface of gold at the bottom of the wells). In all the experiments done so far with these chips, after a week in culture less than half the wells had neurons in them. It is unclear whether these losses were due to neuron death, neuron migration out of the wells, or both. New studies will explore this phenomenon more closely.

Fabrication

A new batch of dummy probes for *in vivo* experiments was fabricated in this period. They were made according to the fabrication procedure which was described in the previous report. The only difference was an improved front-to-back alignment (performed by means of a sandwiched-mask tool). It eased the snapping of the probes from the substrate, and (as demonstrated later on) it may have had a positive impact on the mechanical strength of the probes.

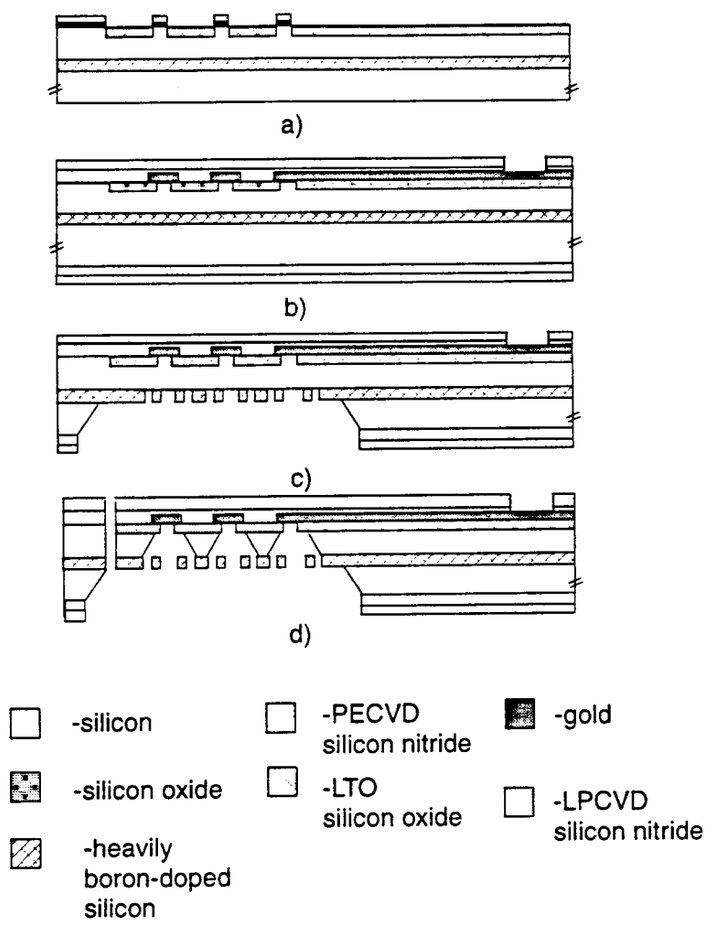
Many of the previously fabricated batch of probes unfortunately broke during the transportation from California to New Jersey. This raised questions about the mechanical strength of the probes. We tested the new batch by loading the tip of a probe with a known force and measuring the corresponding deflection . The figure below shows a probe under test.



We examined probes with or without neuron wells, as well as probes which were briefly dipped in an isotropic etchant to alleviate stress. All these probes had approximately the same stiffness within the measurement error (1.2 ± 0.1N/m), which suggested that the presence of the neuron wells doesn't have a significant impact on the mechanical strength of the probe.

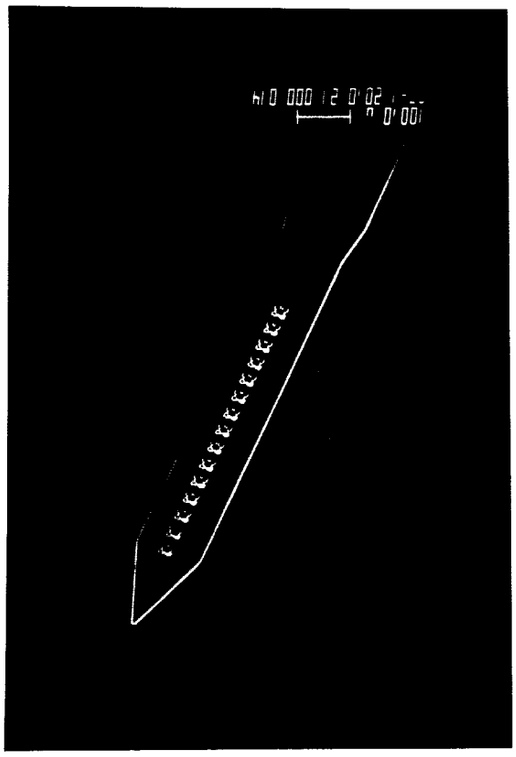
We couldn't succeed in breaking the probe in our set-up, because it was too flexible to maintain the force required to get to the breaking point. According to our experiments, we think that the probe is strong enough, especially after the additional front-to-back alignment step mentioned at the beginning of the text. We plan to do buckling tests, as well as side loading tests to confirm it.

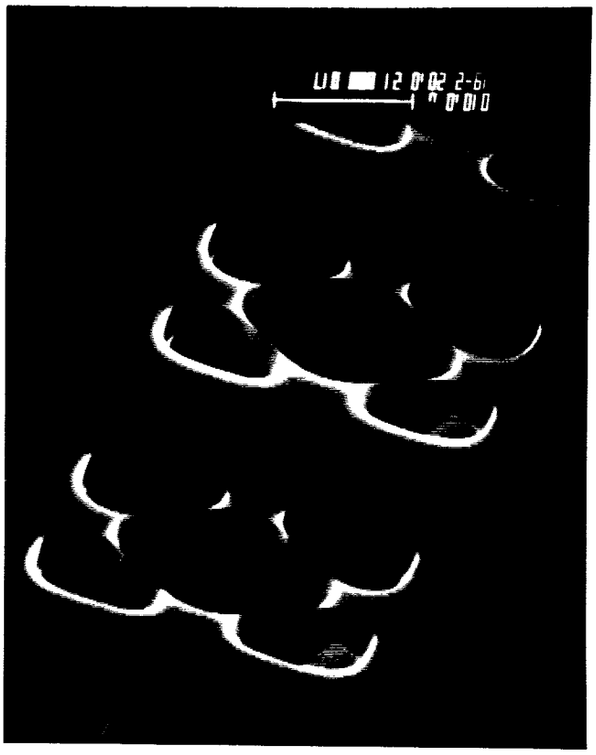
Finally, we want to report about a major breakthrough: the first complete neuroprobes with electrodes have been fabricated. The process was that explained and proposed in previous reports, as summarized in the figure below:



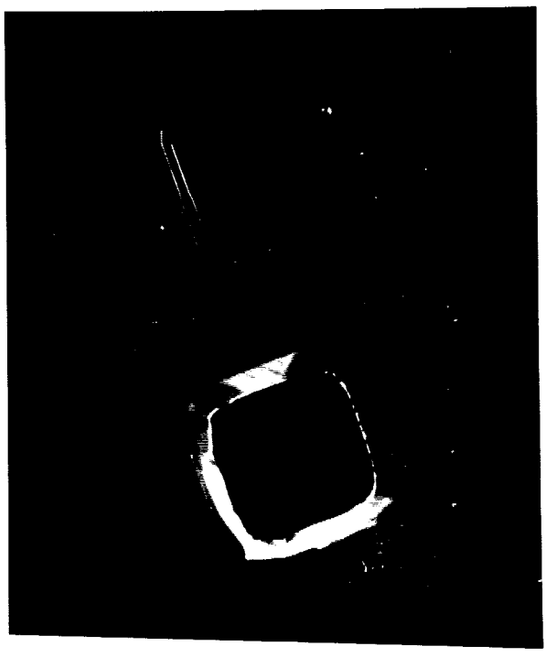
This process promises to be robust enough to enable a good yield (~75%) from the wafer. However, big yield was not achieved with the first wafer because of an error which caused overetching of the grillwork, not a problem with the process itself.

The figures below show a scanning electron micrograph of an entire probe and of a few of the wells. The grillwork was slightly overetched but that will be avoided in the next batch. As can be seen, the grillwork is very thick (3-4 μm, estimated from the picture), so it should be very robust.



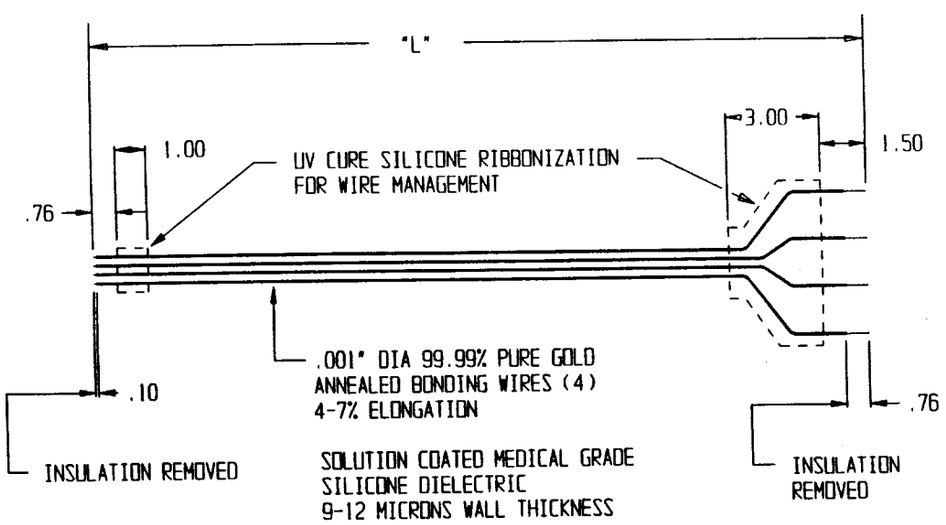


The back side of the probe is shown in the electron micrograph below. The compensation of the probe's corners seems to be fairly good, whereas the front-to-back alignment of the cavity with respect to the front side was made relying on the stepper only, and it is not good enough. (Here, we are talking about rough front-to-back alignment, not about the subsequent fine double-sided alignment for the grillwork mask). In the next generation, a sandwiched mask tool will be used for this kind of alignment.

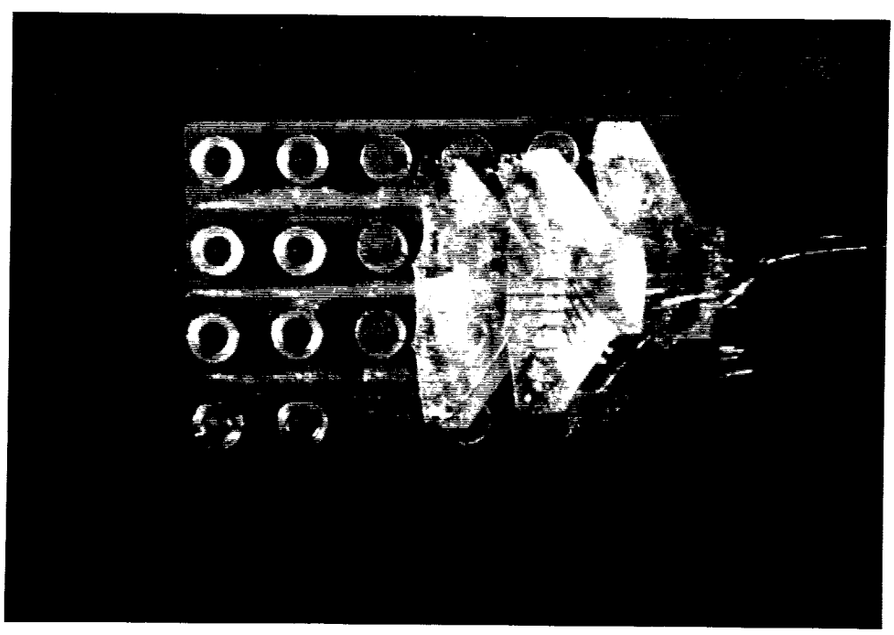


The production of the first generation of complete neuroprobes with electrodes was a very important milestone. New, perfected batches of neuroprobes will be fabricated in the coming quarter.

An additional fabrication issue which was addressed during this quarter was the attachment of sixteen leads from the probe to a skull plug. PI Medical fabricated assemblies for this purpose, made from 25 micron gold wire insulated with approximately 15 microns of MDX4-4210. A diagram is shown below, showing the forming, stripping, and ribbonization. For each probe four assemblies of slightly unequal lengths are needed to provide a match at both ends, with approximately 25 mm. of cable length.



The wires could in principle be ultrasonically bonded, to the probe, but we have chosen to use gold-loaded conducting epoxy. The same material is very convenient to use at the plug end. The photos below show the 16 wire assembly at each end, without overpotting. For the experiments, the bond region will be covered by MDX4-4210.



In Vivo Experiments

1. Moving and survivability of cells in cultured neuron probes

During the past quarter, 9 sessions involving 15 silicon probes were carried out.

The first experiment was done with unstained cells (two probes) and we could not verify the survival of the neurons either in the wells or on the handle area of the probe. No processes were seen outgrowing from the wells 24 hours later and we concluded that the neurons were either dead or failed to grow processes.

In the second experiment, we used the fluorescent LIVE/DEAD TM kit (Molecular Probes) to evaluate the percentage of surviving neurons. This procedure does not require any washing of the cells and allows simultaneous visualization of live and dead cells. In preliminary experiments we found that the optimal concentration for staining is a solution that contains 20 µl Ethidium homodimer and 5 µl calcein dissolved in 10 ml Dulbecco's MEM F-12. It was added to the Dulbecco's solution containing the cell suspension (about 10^6 cells per ml) in a 1:1 dilution. After 30 min of incubation at room temperature live cells (green) and dead cells (red) can be counted.

The next series of experiments were carried out with the cells stained with live/dead reagent. Although about 90% of neurons in the wells were alive (green) right after moving them, it was clear that at least some of the neurons that we moved into the wells were dead prior to any manipulation. This problem can be eliminated by staining neurons prior to moving. For this reason, we are planning to purchase the necessary filter set for our cultured neuron probe setup. Another problem that we encountered was that moving the probes physically when transferring them from one laboratory to the next, some of the cells popped out from the wells. Again, we try to eliminate this problem by carrying out all manipulations in the same lab.

Another major improvement of our system is the installation of a thermostat on the microscope stage that allows continuous carbon dioxide (10%) flow above the culture medium. This procedure can keep pH at a constant level for an extended period of time.

Seven experiments with 13 probes were carried out using the above protocol. Overall, we found that at least 50% of the cells were alive 24 hours after implantation and some cells survived up to 76 hours (the longest observation period). However, the neurons did not grow processes. This was true for cells which were placed into the wells, and also for neurons or neuronal groups on the handle area of the probe. Obviously, this latter observation indicates that the failure to grow processes was not due to the physical manipulation of the cells.

Although it is known that hippocampal neurons do not develop processes in the absence of glial bedding, this is not the case with septal neurons. Since 7 probes were loaded with septal neurons, the causes of failure should be sought in issues other than the lack of glial imbedding. In addition, we have often seen proliferation of neuronal processes in our previous experiments using the same culture media.

To date, we have not identified the cause for the lack of process outgrow. One potential problem is inadequate polylysine treatment of the probe surface. In our next series of experiments we will increase the polylysine concentration. In addition, we will experiment with 10% horse serum added to the MEM, instead of the presently used 10% fetal calf serum.

2. Evaluation of silicon track-induced cell damage

An important issue in this project is the physiological-anatomical conditions of the host environment around the cultured neurons probes. In our previous report we described our silver-staining method for the evaluation of the electroded tracks made acutely or chronically by implanting coated tungsten or stainless steel electrodes. During the last 3 months, we have investigated the histological reaction to chronically implanted "dummy" cultured probes. From 1 to 3 silicon probes were implanted into the dorsal hippocampus of 5 rats. The

probes were placed into the brain under visual control using a dissection microscope. The probes were held either manually by a small forceps or by a custom-designed probe holder. After the probe was lowered into the dorsal hippocampus the shank was broken close to the handle area and the handle was removed. This way the probe shank floated into the brain. The protruding end of the shank was covered by gelfoam and the wound was closed.

The rats were perfused 2 months later. The brains were removed from the skull 24 hours later and were kept in the fixative solution for another week. The area containing the probes were blocked and the coronal sections (100 or 200-m thick) were cut by a vibrotome. The brain was placed with its dorsal surface facing down. In our preliminary experiments, we found that the knife of the vibrotome produces less damage to the probes when the sections are cut from the sharp end of the probe. With such an approach we could recover most probes and the movement of the probes in the brain by the cutting procedure was negligible in most cases. The sections containing the silicon probes and three neighboring sections on each side were stained with the Gallyas method for dark neurons. An example is shown on the page following.

In one rat, bleeding was present around one probe and as a result a small cavity was formed in the hippocampus. In the other rats, dark neurons were observed in the vicinity of only 2 our of 9 probes. In other words, acutely damaged neurons (i.e., within a week prior to sacrifice) around the probes were only very rarely damaged. This lack of recent neuronal damage was characteristic in both hippocampus and the overlying neocortex. Although quantitative comparisons have yet to be made, the number of acutely damaged cells appear significantly less than around similarly positioned metal electrodes (see e.g., our previous quarterly reports). A word of caution is in order, however, since the metal electrodes were fixed to the skull by dental acrylic whereas the silicon probes were freely "floating" in the brain. Whether the reduced damage around the silicon probes is due to the lack of mechanical movement of the probe in the brain or to a better histocompatibility of silicon relative to stainless steel and tungsten has yet to be determined.

In another group of 4 rats, we evaluated the chronic or "cumulative" damage caused by silicon probes using an activated microglia stain, as shown below. Since activated microglia remains in place several weeks and even months after acute or chronic neuronal damage, it is the method of choice for the evaluation of former damage of neurons (Hsu and Buzsaki, 1993). Microglia reaction was present around every probe in every animal. Interestingly, such a reaction was not evident at every level along the probe track. The most frequently affected site was the tip area of the silicon probe and significantly less microglial reaction was seen in the upper parts of the probe. Again, a critical factor responsible for such a reaction could be the larger mechanical movement of the tip of the electrode relative to the upper shank, either during the implantation procedure or at a later time point.